



Noninvasive measurement of gene expression in skeletal muscle

Glenn Walter, Elisabeth R. Barton, and H. Lee Sweeney*

Department of Physiology, University of Pennsylvania, Philadelphia, PA, 19104-6085

Communicated by Mildred Cohn, University of Pennsylvania School of Medicine, Philadelphia, PA, February 16, 2000 (received for review September 29, 1999)

We have developed a noninvasive detection method for expression of viral-mediated gene transfer. A recombinant adenovirus was constructed by using the gene for arginine kinase (AK), which is the invertebrate correlate to the vertebrate ATP-buffering enzyme, creatine kinase. Gene expression was noninvasively monitored using ^{31}P -magnetic resonance spectroscopy (^{31}P -MRS). The product of the AK enzyme, phosphoarginine (PArg), served as an MRS-visible reporter of AK expression. The recombinant adenovirus coding for arginine kinase (rAdCMVAK) was injected into the right hindlimbs of neonatal mice. Two weeks after injection of rAdCMVAK, a unique ^{31}P -MRS resonance was observed. It was observable in all rAdCMVAK injected hindlimbs and was not present in the contralateral control or the vehicle injected limb. PArg and phosphocreatine (PCr) concentrations were calculated to be 11.6 ± 0.90 and 13.6 ± 1.1 mM respectively in rAdCMVAK injected limbs. AK activity was demonstrated *in vivo* by monitoring the decreases in PArg and ATP resonances during prolonged ischemia. After 1 h of ischemia intracellular pH was 6.73 ± 0.06 , PCr/ATP was decreased by $77 \pm 8\%$, whereas PArg/ATP was decreased by $50 \pm 15\%$ of basal levels. PArg and PCr returned to basal levels within 5 min of the restoration of blood flow. AK activity persisted for at least 8 mo after injection, indicating that adenoviral-mediated gene transfer can produce stable expression for long periods of time. Therefore, the cDNA encoding AK provides a useful reporter gene that allows noninvasive and repeated monitoring of gene expression after viral mediated gene transfer to muscle.

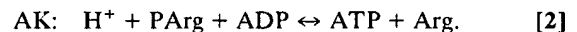
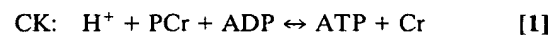
nuclear magnetic resonance | gene therapy | adenovirus | creatine kinase | arginine kinase

Recombinant viruses provide highly efficient vehicles for the delivery of transgenes to both skeletal and cardiac muscle (1, 2). Recombinant adenoviruses have been used to replace missing proteins in mouse models of muscular dystrophies (3, 4), as well as to create muscles that secrete therapeutic proteins (2, 5, 6). Previous studies have shown that direct injection of recombinant adenovirus into neonatal murine muscle can result in long-term, efficient expression in $>60\%$ of myofibers (7). However, there is tremendous variability in the extent and length of expression. If gene therapy in humans becomes a reality, then monitoring of gene transfer efficiency and expression in clinical settings would currently require invasive techniques (8). Therefore, an obvious need exists for noninvasive tools to measure the efficacy of gene transfer. Despite the recent advent of new methods to image gene expression *in vivo* in transparent tissues (9, 10) and using positron emitting radioisotopes (11), MRI, and magnetic resonance spectroscopy (MRS) have the greatest potential for use in noninvasive gene transfer assessment. MRS is a classic tool for performing noninvasive measurements of metabolic status of tissues without the use of ionizing or nephrotic agents. MRS has the added advantage that spectral information can be obtained in seconds and can be used to determine enzymatic rates *in vivo* quantitatively.

An ideal reporter gene must satisfy a number of criteria. First, it must provide a unique signal so that transgene expression can

be monitored against a background of natively expressed genes. Second, it must be small enough so that it can be expressed with therapeutic genes in bicistronic constructs so that it serves as a direct marker for the entire virus. Third, it must not interfere with normal tissue function. In our approach, we have constructed an adenovirus expressing arginine kinase (AK) that satisfies the criteria listed above.

Creatine kinase (CK) catalyzes a near-equilibrium, dead end reaction in a variety of vertebrate tissues (primarily nerve and muscle). An analogous reaction that provides buffering in invertebrate tissue is catalyzed by AK:



The equilibrium constant for the CK (Eq. 1) reaction is approximately 166, whereas under the same conditions it is approximately 10-fold less for AK (Eq. 2) (12, 13). Measurements of the levels of free Arg in the cytoplasm of mammalian muscle range from 0.1 to 1.0 mM (14). Thus, we anticipated that if AK was introduced into mammalian muscle, the muscle would produce phosphoarginine (PArg) at equilibrium concentrations in the range of 3–30 mM. Based on previous studies on invertebrate muscle (12, 15, 16), PArg would provide a unique phosphorus NMR signal of magnitude and position that could be easily recognized in vertebrate muscle. In addition, we hypothesized that the combined expression of AK and CK could be beneficial to the cell under conditions of low ATP/adenosine 5'-diphosphate and intracellular pH (12). In this study, AK expression was achieved in the hindlimb muscles of mice by the direct injection of a recombinant adenovirus encoding for an AK cDNA (rAdCMVAK). AK expression was monitored noninvasively by using ^{31}P -magnetic resonance spectroscopy (^{31}P -MRS). Herein, we demonstrate that both the expression and the activity of the AK transgene can be measured noninvasively and followed as a function of time with ^{31}P -MRS.

Methods

Viral Construct. The cDNA for *Drosophila melanogaster* AK was amplified from an adult *Drosophila* cDNA library (5'-Stretch, CLONTECH) using the PCR (sense strand, CGCCCTTTTA-CAATGGTCAAT; antisense strand, GATTGTTGCGTAT-GCCCCGAA). The cDNA fragment was then ligated into the *Bam*HI–*Hin*DIII sites of the pAdLox vector (17). Expression was driven by CMV promoter and was stabilized by a simian virus 40

Abbreviations: RT-PCR, reverse transcriptase-PCR; PCr, phosphocreatine; PArg, phosphoarginine; rAdCMVAK, adenovirus coding for arginine kinase; ppm, parts per million; MRS, magnetic resonance spectroscopy; EDL, extensor digitorum longus; CMV, cytomegalovirus; AK, arginine kinase; CK, creatine kinase.

*To whom reprint requests should be addressed. e-mail: lsweeney@mail.med.upenn.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

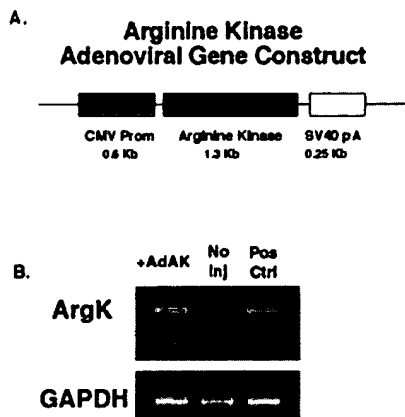


Fig. 1. (A) Schematic diagram for rAdCMVAK gene construct. Expression was driven by nonspecific CMV promoter and was stabilized by an simian virus 40 polyadenylation sequence (SV40pA). From this construct, a Δ E1– Δ E3 adenovirus was prepared by using published methods (17). (B) RT-PCR was used to detect the presence of AK transcripts in rAdCMVAK-injected muscles. Total RNA isolated from frozen tissue was subjected to RT-PCR by using oligonucleotides specific for *Drosophila* AK and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). AK transcripts were detected in rAdCMVAK-injected muscles (+AdAK) but not in the contralateral control (No Inj). AK and GAPDH primers served as positive controls for the procedure (Pos Ctrl).

polyadenylation sequence (SV40pA). Fig. 1 shows a schematic for this gene construct. An Δ E1– Δ E3 adenovirus was prepared from the final construct (rAdCMVAK) by using published methods (17) by the University of North Carolina Vector Core.

Injections. All experiments involving animals were approved by the University of Pennsylvania's Animal Care and Use Committee. Forty microliters of 10% glycerol/PBS containing approximately 10^{10} adenoviral particles were injected into the interstitial space of the anterior and posterior muscle compartment of the right hindlimb of anesthetized C57BL/6 neonatal mice between 1 and 3 days of age. Once the mice regained consciousness, they were returned to the animal facility until further study.

Detection of Transgene Expression. The reverse transcriptase–PCR (RT-PCR) was used to detect the presence of AK transcripts in rAdCMVAK-injected muscles. Total RNA was isolated from frozen tissue using a commercial kit (RNAqueous, Ambion, Austin, TX) and was subjected to reverse transcription and PCR (Perkin–Elmer) using oligonucleotides specific for *Drosophila* AK (sense primer, TGCCGAGGCTTACACAG; antisense primer, AAGTGGTCGTCGATCAG). Primers that amplified glyceraldehyde 3-phosphate dehydrogenase (sense primer, TGAAGGTCGGAGTCAACGGATTTGGT; antisense primer, CATGTGGGCCATGAGGTCCACCAC) served as a positive control for the procedure.

NMR. High resolution NMR spectra were recorded from mouse hindlimbs using a 5-mm diameter surface coil double tuned to ^1H (300 MHz) and ^{31}P (121 MHz) on a Bruker 300 MHz AMX spectrometer. Mice were anesthetized and secured to a home built vertical probe such that the surface coil could be positioned over the anterior portion of the lower hindlimb. Magnetic field homogeneity was adjusted using the free proton signal, resulting in a typical full width half maximum of 0.2 parts per million (ppm). ^{31}P -MRS spectra were obtained with a pulse repetition time of 5.4 s, a pulse width of 20 micro seconds, a spectral width of 12,000 Hz, and 4,096 complex data points. Peak areas, chemical shifts, and line widths were measured by using time

domain analysis. All chemical shifts were determined relative to the phosphocreatine (PCr) resonance. Intracellular pH was determined based on the P_i chemical shift.

Basal high energy phosphate levels were measured in the mouse hindlimbs starting at 2 wk after injection of rAdCMVAK and continued until 8 mo after injection. AK activity was detected *in vivo* by monitoring the degradation of PArg levels during circulatory occlusion. Prolonged ischemia was produced by the application of a tourniquet around the upper thigh muscles.

In Vitro Force Measurement. The extensor digitorum longus (EDL) was removed from the hind limb, retaining the proximal and distal tendon. The intact muscle was immersed in a Ringer's solution buffered to pH 7.4 with 25 mM Hepes, which was continuously oxygenated and maintained at $25 \pm 0.5^\circ\text{C}$. Muscles were mounted horizontally in the muscle bath, attached by tendinous insertions to a post at one end and to the lever of a dual mode servomotor system at the other. Muscle length was adjusted to the length (L_0) at which maximal twitch force is reached. Stimulation was delivered via two platinum plate electrodes, which were positioned along the length of the muscle. The maximal tetanic force was determined by using 120 Hz, 500-ms supramaximal pulses.

Results

MRS Measurement of AK Expression in Skeletal Muscle. Solution experiments. To determine the feasibility of measuring AK activity in vertebrate muscle a solution of the expected physiological concentrations of ATP, P_i , PCr, and PArg was constructed. As seen in Fig. 2, PArg was well resolved from PCr and γ ATP with a chemical shift of 0.47 ppm relative to PCr. In addition, the solution was used to determine the appropriate spectral analysis package to analyze both phantom and mouse skeletal muscle data with the minimal amount of user bias. Based on these preliminary studies a time domain Hankel single value decomposition algorithm (18) was used (Fig. 2) and user peak selection was avoided.

Detection of AK Expression in Skeletal Muscle by NMR. Due to the small volume of muscle in the neonatal mouse hindlimb (1- to 5-day-old), the earliest possible ^{31}P -MRS measurements were made 12–13 days after gene delivery. At this time point, a unique resonance was observed at 0.49 ppm relative to the PCr resonance. This resonance at 0.49 ± 0.01 ($n = 24$) ppm away from the PCr resonance was observable in all of the injected limbs and was not present in the contralateral control leg or vehicle-injected limbs (Fig. 3). The presence of AK transcripts in rAdCMVAK injected muscles was confirmed by RT-PCR (Fig. 1B). The PArg resonance was observed in rAdCMVAK muscles up to 8 mo after gene delivery, indicating persistent expression of AK. The chemical shift of PCr relative to γ ATP was 2.46 ± 0.02 ppm, was not different between experimental groups, and was the same as previously reported in murine muscle (19). Both PCr and PArg existed as single, well-defined Lorentzian resonances with line widths of 44 ± 2.7 Hz and 32 ± 2.0 Hz, respectively.

The PArg/ γ ATP ratio in the experimental hindlimb was 1.25 ± 0.12 whereas the PCr/ γ ATP was 1.31 ± 0.12 . The total PCr + PArg/ γ ATP was 2.56 ± 0.16 in the rAdCMVAK-injected leg and PCr/ γ ATP was 2.65 ± 0.58 in control limbs. To calculate absolute PCr and PArg concentration, perchloric acid extracts of injected and uninjected EDLs were used to determine ATP levels by high performance liquid chromatography (20). Based on the measured ATP value of 8.7 mM in the injected limb and following correction for saturation, PArg and PCr were calculated to be 11.6 ± 0.85 and 13.6 ± 1.1 mM in the rAdCMVAK-injected muscles. Using the combined equilibrium of CK and

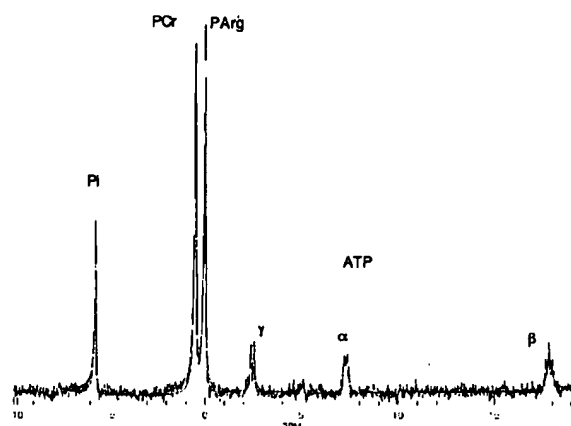


Fig. 2. ^{31}P -MRS spectra of a model solution containing expected physiological concentrations of ATP, P_i , PCr, and PArg. The model solution consisted of 30 mM PCr, 10 mM P_i , 30 mM PArg, and 8 mM ATP in 0.4 ml of distilled water. In solution, PArg was well resolved from PCr and γATP with a chemical shift of 0.47 ppm relative to PCr. The model solution was used to determine the appropriate spectral analysis package for the analysis of both phantom and mouse skeletal muscle data with the minimal amount of user bias. The solid line is the best fit to the free induction decay using a Hankel single value decomposition algorithm (18), which avoided peak selection and the use of previous knowledge.

AK, free Arg levels were calculated to range from 0.3 to 0.6 mM in the rAdCMVAK-injected muscles.

Detection of AK Activity in Skeletal Muscle. AK activity was determined *in vivo* by monitoring the decreases in PArg, PCr, and ATP resonances during prolonged ischemia (Fig. 4). After 1 h of ischemia intracellular pH was 6.73 ± 0.06 , PCr/ATP decreased by $77 \pm 8\%$, whereas PArg/ATP decreased by $50 \pm 15\%$ of basal levels ($n = 6$). PArg was completely resynthesized within 5 min of the restoration of blood flow. PArg depletion was demonstrated up to 8 mo in rAdCMVAK muscle. These results demonstrate AK activity and the equilibration of the PArg pool with cytoplasmic ATP.

In Vitro Mechanics. The expression of AK in the EDL did not have a detrimental effect on maximal force production. The EDLs of 6-mo-old mice ($n = 3$) were removed for the determination of

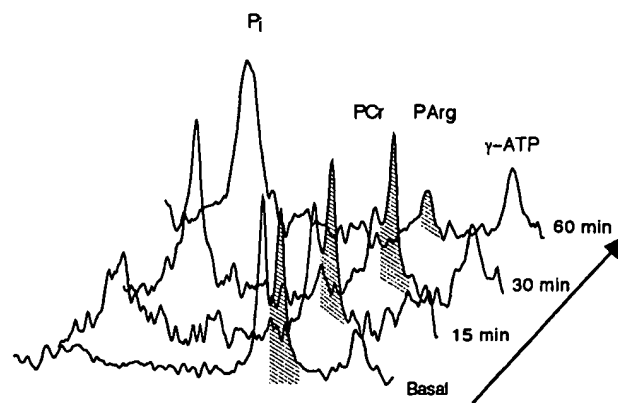


Fig. 4. Stack plot of changes in high energy phosphates during prolonged ischemia in the rAdCMVAK-injected limb. During circulatory occlusion, PCr levels are depleted and inorganic P_i levels rise within 15 min of ischemia followed by the depletion of PArg.

in vitro maximal tetanic muscle force. There was no significant difference in maximal tetanic force between rAdCMVAK and the contralateral control EDL muscles. Furthermore, the specific forces were not different from uninjected control EDLs.

Discussion

We have shown that AK can serve as a noninvasive monitoring system for viral-mediated gene delivery. This transgene upholds a number of criteria necessary for a gene marker in that it is small, nontoxic, and unique against the mammalian background. In addition, the expression of this particular marker in striated muscle introduces an additional thermodynamic buffer into a highly energetic tissue. This can provide insight into the role of phosphagen kinases in cellular function.

The general implication of these results are twofold. First, AK can be used as a noninvasive marker for gene transfer in vertebrate skeletal muscles. Secondly, skeletal muscles that express a combination of CK and AK should have an extended thermodynamic buffering range (12, 21). The CK equilibrium is poised to buffer ATP levels during the initial burst of ATPase activity, whereas AK buffering is greater under low ATP and intracellular pH levels.

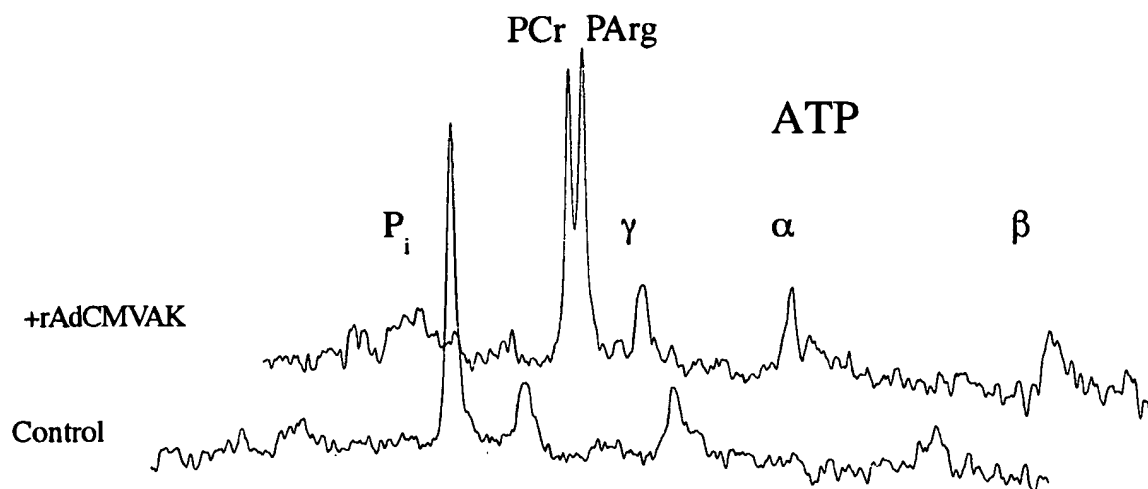


Fig. 3. *In vivo* basal ^{31}P spectra from the hindlimbs of a 6-mo-old mouse. ^{31}P -MRS spectra from the rAdCMVAK-injected limb (Upper spectrum) reveal a ^{31}P resonance at the chemical shift for PArg that is not present in the contralateral control limb (Lower spectrum).

AK as a Noninvasive Marker Gene. The use of a MRS-visible marker has the obvious clinical benefit of monitoring transgene expression without removing the tissue from the subject. Adenoviruses are known to infect nonreplicating cell types such as terminal-differentiated myocytes. Muscle has been shown to be an easy target for adenoviral gene transfer *in vivo*, rendering muscle a viable tissue for gene therapy and the endogenous production of therapeutic secreted proteins (2, 5, 6). In this study, an adenovirus was injected into neonates to avoid an immune response that normally occurs in adult muscle (7). With the advent of high titer recombinant adeno-associated viruses, which lack immunogenic response, the prospect of human gene therapy using adeno-associated viruses is becoming a reality (2, 3, 6). Because of the large volume of tissues necessary to target in humans, methods need to be developed to assess the degree of gene transfer achieved. Moreover, because of the possibility that only transient expression (22) of the therapeutic gene is achieved, this method should be nondamaging, nontoxic, and capable of sequential measures. Whereas MR strategies to monitor gene expression have relied on receptor-mediated or probe activation strategies (23–26), xenografts (27), and the expression of an endogenous gene (28), here, we have presented a noninvasive MRS method capable of directly monitoring the expression of a unique, nontoxic gene marker *in vivo*. This method has the advantage that when tissue specificity or when inducible expression is desired, the reporter and therapeutic gene are under control of the same promoter. Furthermore, this method is not hampered by indicator delivery limitations. We demonstrated that AK expression in murine muscle resulted in the production of a unique resonance noninvasively observable with ^{31}P -MRS. This resonance cannot be explained as a shift in the PCr peak or by changes in line width because it remained a well-defined, narrow resonance 2.46 ppm upfield from the γ -ATP in all limbs as previously reported (19). Due to the high concentration (≈ 12 mM) and the large chemical shift of PArg compared to other ^{31}P -MRS visible metabolites, it was possible to repeatedly monitor the transgene product (PArg) and AK enzyme activity *in vivo*. Future experiments will be aimed at using saturation transfer studies to determine the simultaneous flux through CK and AK at rest and during exercise and its relationship to AK content (29, 30).

Energetic Consequences of AK Expression. We have focused on AK as a noninvasive marker for gene transfer, but AK may prove to have an additional benefit to transiently ischemic muscle. We have added an additional cytoplasmic ATP buffer to vertebrate skeletal muscle by using an invertebrate transgene. The CK system is poised to keep the ATP/adenosine 5'-diphosphate at very high levels, enabling high ATPase fluxes at the onset of burst activity at the cost of PCr. Unfortunately, PCr cannot continue to sustain its thermodynamic buffer capacity at low ATP/adenosine 5'-diphosphate ratios that exist during sustained activity or prolonged ischemia. Ultimately, ATP levels are depleted, rigor ensues, and Ca^{2+} accumulation occurs in the sarcoplasm resulting in the loss of membrane integrity and cell death. On the other hand, invertebrate phosphagens exist that could also serve to buffer ATP utilization at low PCr values (12). Based on the difference in the CK and AK equilibrium constants, if the two enzymes exist in the same cell, then the initial

flux through the AK reaction would be small compared with that through CK (12). As PCr is depleted, the ATPase flux will be primarily supported by AK. Thus the coexpression of AK and CK should prove beneficial to skeletal cells under conditions of prolonged ischemia or fatiguing conditions. Following the introduction of AK into the mammalian muscle cytoplasm a large PArg pool is expected to be formed without disturbing the normal levels of ATP or PCr (13). However, upon depletion of PCr which would occur in ischemia, CK can no longer buffer changes in ATP. At this point, the PArg pool will continue to buffer changes in ATP levels. In addition, the AK reaction will tend to slow the fall of pH. Direct support for this hypothesis is the observation that vertebrate muscle is known to take up the synthetic creatine analog cyclocreatine at high concentrations. When cyclocreatine is the substrate the CK equilibrium is reduced to 3% of that when native Cr is the substrate. Cyclocreatine also has been shown to delay ischemia-induced damage in both skeletal and cardiac muscle (21, 31). AK also buffers the free arginine pool and unlike creatine can be used by a number of different pathways. Of primary interest is whether transient increases in free arginine and/or PArg effect NO production and muscle function.

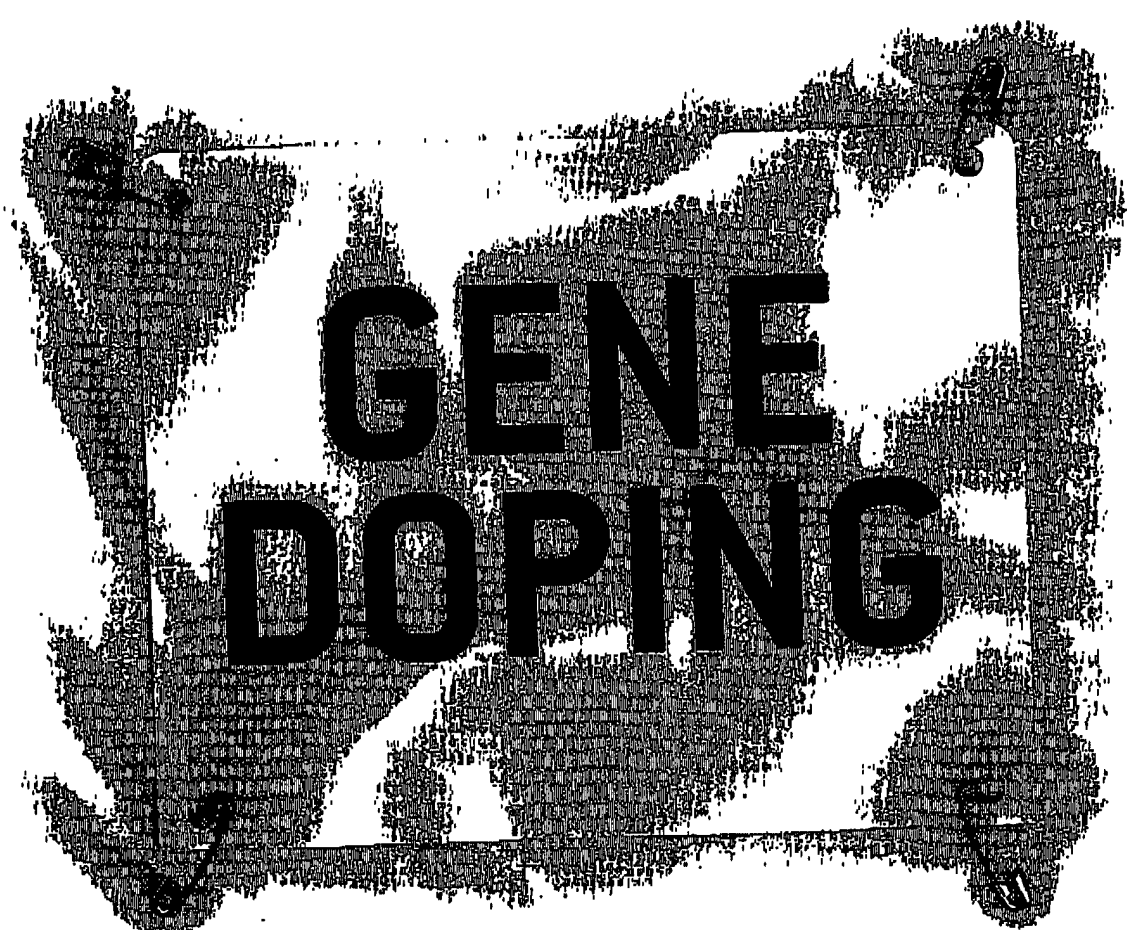
Implications for Gene Therapy. We have verified that adenovirus gene delivery in newborn mice can result in persistent expression of the foreign genetic material for at least 8 mo. ^{31}P -MRS offers the ability to rapidly and repeatedly monitor AK expression in the same muscle permitting the study of the influence of growth, aging, and disease status on gene expression. The coding sequence for AK is small enough (1.3 kb) that it can be coexpressed with growth and angiogenic factors and other small proteins in adeno-associated virus expression systems (32), which are limited in packing size (4.4 kb). These results demonstrate the presence of PArg in the unlocalized ^{31}P -MRS spectra of small muscles (<30 mg); however, in larger muscles heterogeneity in gene expression throughout the muscle is expected due to a lack of global delivery. Therefore, future experiments will be geared toward providing localized information by the direct imaging of PArg and PCr levels spatially. Recent advances in MRI technology have permitted the imaging of PCr, ATP, and P_i in the human forearm muscles by using a clinical MRI scanner (33). Our preliminary measurements have demonstrated the feasibility of such a technique to image regional (4-ml volumes of interest) PCr content in the rabbit hindlimb in <8 min. Current experiments are aimed at producing the spectral selectivity necessary to image both PCr and PArg in the rabbit hindlimb expressing AK. AK as a gene marker is not limited to skeletal muscle, it could be applied in any tissues in which the free arginine levels are above 0.2 mM such that the resulting PArg resonance is above the noise limit. This approach has previously been shown to be successful by the expression of CK in the transgenic mouse liver (28).

We would like to acknowledge the help of Rudy Stuppard for the HPLC measurements, Daria Shoturma for her expert technical assistance, and the University of North Carolina Vector Core for the preparation of rAdCMVAK virus. This work was supported by grants from the National Institutes of Health (AR/NS43648) and the Muscular Dystrophy Association.

1. Woo, Y. J., Zhang, J. C., Vijayasathay, C., Zwacka, R. M., Englehardt, J. F., Gardner, T. J. & Sweeney, H. L. (1998) *Circulation* **98**, II255–II261.
2. Barton-Davis, E. R., Shoturma, D. I., Musaro, A., Rosenthal, N. & Sweeney, H. L. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 15603–15607.
3. Haecker, S. E., Stedman, H. H., Balice-Gordon, R. J., Smith, D. B., Greelish, J. P., Mitchell, M. A., Wells, A., Sweeney, H. L. & Wilson, J. M. (1996) *Hum. Gene Ther.* **7**, 1907–1914.
4. Ragot, T., Vincent, N., Chafey, P., Vigne, E., Gilgenkrantz, H., Couton, D.,

- Cartaud, J., Briand, P., Kaplan, J. C., Perricaudet, M., et al. (1993) *Nature (London)* **361**, 647–650.
5. Rivard, A., Silver, M., Chen, D., Kearney, M., Magner, M., Annex, B., Peters, K. & Isner, J. M. (1999) *Am. J. Pathol.* **154**, 355–363.
6. Gao, G. P., Qu, G., Faust, L. Z., Engdahl, R. K., Xiao, W., Hughes, J. V., Zoltick, P. W. & Wilson, J. M. (1998) *Hum. Gene Ther.* **9**, 2353–2362.
7. Stratford-Perricaudet, L. D., Makeh, I., Perricaudet, M. & Briand, P. (1992) *J. Clin. Invest.* **90**, 626–630.

8. Gussoni, E., Blau, H. M. & Kunkel, L. M. (1997) *Nat. Med.* **3**, 970–977.
9. Misteli, T. & Spector, D. L. (1997) *Nat. Biotechnol.* **15**, 961–964.
10. Jacobs, W. R., Jr., Barletta, R. G., Udani, R., Chan, J., Kalkut, G., Sosne, G., Kieser, T., Sarkis, G. J., Hatfull, G. F. & Bloom, B. R. (1993) *Science* **260**, 819–822.
11. Gambhir, S. S., Barrio, J. R., Phelps, M. E., Iyer, M., Namavari, M., Satyamurthy, N., Wu, L., Green, L. A., Bauer, E., MacLaren, D. C., *et al.* (1999) *Proc. Natl. Acad. Sci. USA* **96**, 2333–2338.
12. Ellington, W. R. (1989) *J. Exp. Biol.* **143**, 177–194.
13. Sweeney, H. L. (1994) *Med. Sci. Sports Exer.* **26**, 30–36.
14. Bergström, J., Alvestrand, A., Fürst, P., Hultman, E., Sahlin, K., Vinnars, E. & Widström, A. (1976) *Clin. Sci. Mol. Med.* **51**, 589–599.
15. Rao, B. D., Buttlair, D. H. & Cohn, M. (1976) *J. Biol. Chem.* **251**, 6981–6986.
16. Nageswara Rao, B. D. & Cohn, M. (1981) *J. Biol. Chem.* **256**, 1716–1721.
17. Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y. & Phipps, M. L. (1997) *J. Virol.* **71**, 1842–1849.
18. van den Boogaart, A., Howe, F. A., Rodrigues, L. M., Stubbs, M. & Griffiths, J. R. (1995) *NMR Biomed.* **8**, 87–93.
19. Steeghs, K., Benders, A., Oerlemans, F., de Haan, A., Heerschap, A., Ruitenbeck, W., Jost, C., van Deursen, J., Perryman, B., Pette, D., *et al.* (1997) *Cell* **89**, 93–103.
20. Wiseman, R. W., Moerland, T. S., Chase, P. B., Stuppard, R. & Kushmerick, M. J. (1992) *Anal. Biochem.* **204**, 383–389.
21. Roberts, J. J. & Walker, J. B. (1982) *Am. J. Physiol.* **243**, H911–H916.
22. Ye, X., Rivera, V. M., Zoltick, P., Cerasoli, F., Jr., Schnell, M. A., Gao, G., Hughes, J. V., Gilman, M. & Wilson, J. M. (1999) *Science* **283**, 88–91.
23. Bogdanov, A., Jr. & Weissleder, R. (1998) *Trends Biotechnol.* **16**, 5–10.
24. Moore, A., Basilion, J. P., Chiocca, E. A. & Weissleder, R. (1998) *Biochim. Biophys. Acta* **1402**, 239–249.
25. de Marco, G., Bogdanov, A., Marecos, E., Moore, A., Simonova, M. & Weissleder, R. (1998) *Radiology* **208**, 65–71.
26. Moats, R. F., Fraser, S. E. & Mead, T. J. (1997) *Angew. Chem. Int. Ed. Engl.* **36**, 726–727.
27. Stegman, L. D., Themetulla, A., Beattie, B., Kievit, E., Lawrence, T. S., Blasberg, R. G. Tjuvajev, J. G. & Ross, B. D. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 9821–9826.
28. Koretsky, A. P., Bronsan, M. J., Chen, L. H., Chen, J. D. & Van Dyke, T. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3112–3116.
29. Meyer, R. A., Kuchmerick, M. J. & Brown, T. R. (1982) *Am. J. Physiol.* **242**, C1–C11.
30. Bittl, J. A., DeLayrc, J. & Ingwall, J. S. (1987) *Biochemistry* **26**, 6083–6090.
31. Osbakken, M., Ito, K., Zhang, D., Ponomarenko, I., Ivanics, T., Jahngen, E. G. & Cohn, M. (1992) *Cardiology* **80**, 184–195.
32. Rabinowitz, J. E. & Salmulski, J. (1998) *Curr. Opin. Biotech.* **9**, 470–475.
33. Greenman, R. L., Elliott, M. A., Vandenborne, K., Schnall, M. D. & Lenkinski, R. E. (1998) *Magn. Reson. Med.* **39**, 851–854.



GENE DOPING

Gene therapy for restoring muscle lost to age or disease is poised to enter the clinic, but elite athletes are eyeing it to enhance performance.

Can it be long before gene doping changes the nature of sport?

By H. Lee Sweeney

Athletes will be going to Athens next month to take part in a tradition begun in Greece more than 2,000 years ago. As the world's finest specimens of fitness test the extreme limits of human strength, speed and agility, some of them will probably also engage in a more recent, less inspiring Olympic tradition: using performance-enhancing drugs. Despite repeated scandals, doping has become irresistible to many athletes, if only to keep pace with competitors who are doing it. Where winning is paramount, athletes will seize any opportunity to gain an extra few split seconds of speed or a small boost in endurance.

Sports authorities fear that a new form of doping will be undetectable and thus much less preventable. Treatments that regenerate muscle, increase its strength, and protect it from degra-

dation will soon be entering human clinical trials for muscle-wasting disorders. Among these are therapies that give patients a synthetic gene, which can last for years, producing high amounts of naturally occurring muscle-building chemicals.

This kind of gene therapy could transform the lives of the elderly and people with muscular dystrophy. Unfortunately, it is also a dream come true for an athlete bent on doping. The chemicals are indistinguishable from their natural counterparts and are only generated locally in the muscle tissue. Nothing enters the bloodstream, so officials will have nothing to detect in a blood or urine test. The World Anti-Doping Agency (WADA) has already asked scientists to help find ways to prevent gene therapy from becoming the newest means of doping. But as these treatments enter clinical trials and, eventually, widespread use, preventing athletes from gaining access to them could become impossible.

Is gene therapy going to form the basis of high-tech cheating in athletics? It is certainly possible. Will there be a time

ATHLETES BUILD MUSCLE through intensive training. This Olympic-class rower's back displays the result of his hard work. But gene therapy could allow athletes to build more muscle, faster, and to stay strong longer without further effort.

when gene therapy becomes so commonplace for disease that manipulating genes to enhance performance will become universally accepted? Perhaps. Either way, the world may be about to watch one of its last Olympic Games without genetically enhanced athletes.

Loss Leads to Gain

RESEARCH TOWARD genetically enhancing muscle size and strength did not start out to serve the elite athlete. My own work began with observing members of my family, many of whom lived well into their 80s and 90s. Although they enjoyed

in all mammals and probably results from a cumulative failure to repair damage caused by normal use. Intriguingly, aging-related changes in skeletal muscle resemble the functional and physical changes seen in a suite of diseases collectively known as muscular dystrophy, albeit at a much slower rate.

In the most common and most severe version of MD—Duchenne muscular dystrophy—an inherited gene mutation results in the absence of a protein called dystrophin that protects muscle fibers from injury by the force they exert during regular movement. Muscles are good at re-

lation between muscle size and its activity saves energy. Skeletal muscle is exquisitely tuned to changing functional demands. Just as it withers with disuse, it grows in size, or hypertrophies, in response to repeated exertions. The increased load triggers a number of signaling pathways that lead to the addition of new cellular components within individual muscle fibers, changes in fiber type and, in extreme conditions, addition of new muscle fibers.

To be able to influence muscle growth, scientists are piecing together the molecular details of how muscle is naturally built and lost. Unlike the typical cell whose membrane contains liquid cytoplasm and a single nucleus, muscle cells are actually long cylinders, with multiple nuclei, and cytoplasm consisting of still more long tiny fibers called myofibrils [see box on opposite page]. These myofibrils, in turn, are made of stacks of contractile units called sarcomeres. Collectively, their shortening produces muscle contractions, but the force they generate can damage the muscle fiber unless it is channeled outward. Dystrophin, the protein missing in Duchenne muscular dystrophy patients, conducts this energy across the muscle cell's membrane, protecting the fiber.

Yet even with dystrophin's buffering, muscle fibers are still injured by normal use. In fact, that is believed to be one way that exercise builds muscle mass and strength. Microscopic tears in the fibers caused by the exertion set off a chemical alarm that triggers tissue regeneration, which in muscle does not mean production of new muscle fibers but rather repairing the outer membrane of existing fibers and plumping their interior with new myofibrils. Manufacturing this new protein requires activation of the relevant genes within the muscle cell's nuclei, and when the demand for myofibrils is great, additional nuclei are needed to bolster the muscle cell's manufacturing capacity.

Local satellite cells residing outside the muscle fibers answer this call. First these muscle-specific stem cells proliferate by normal cell division, then some of their progeny fuse with the muscle fiber,

Raising IGF-I allows us to break the connection between muscle use and its size.

generally good health, their quality of life suffered because of the weakness associated with aging. Both muscle strength and mass can decrease by as much as a third between the ages of 30 and 80.

There are actually three types of muscle in the body: smooth muscle, lining internal cavities such as the digestive tract; cardiac muscle in the heart; and skeletal muscle, the type most of us think of when we think of muscle. Skeletal muscle constitutes the largest organ of the body, and it is this type—particularly the strongest so-called fast fibers—that declines with age. With this loss of strength, losing one's balance is more likely and catching oneself before falling becomes more difficult. Once a fall causes a hip fracture or other serious injury, mobility is gone completely.

Skeletal muscle loss occurs with age

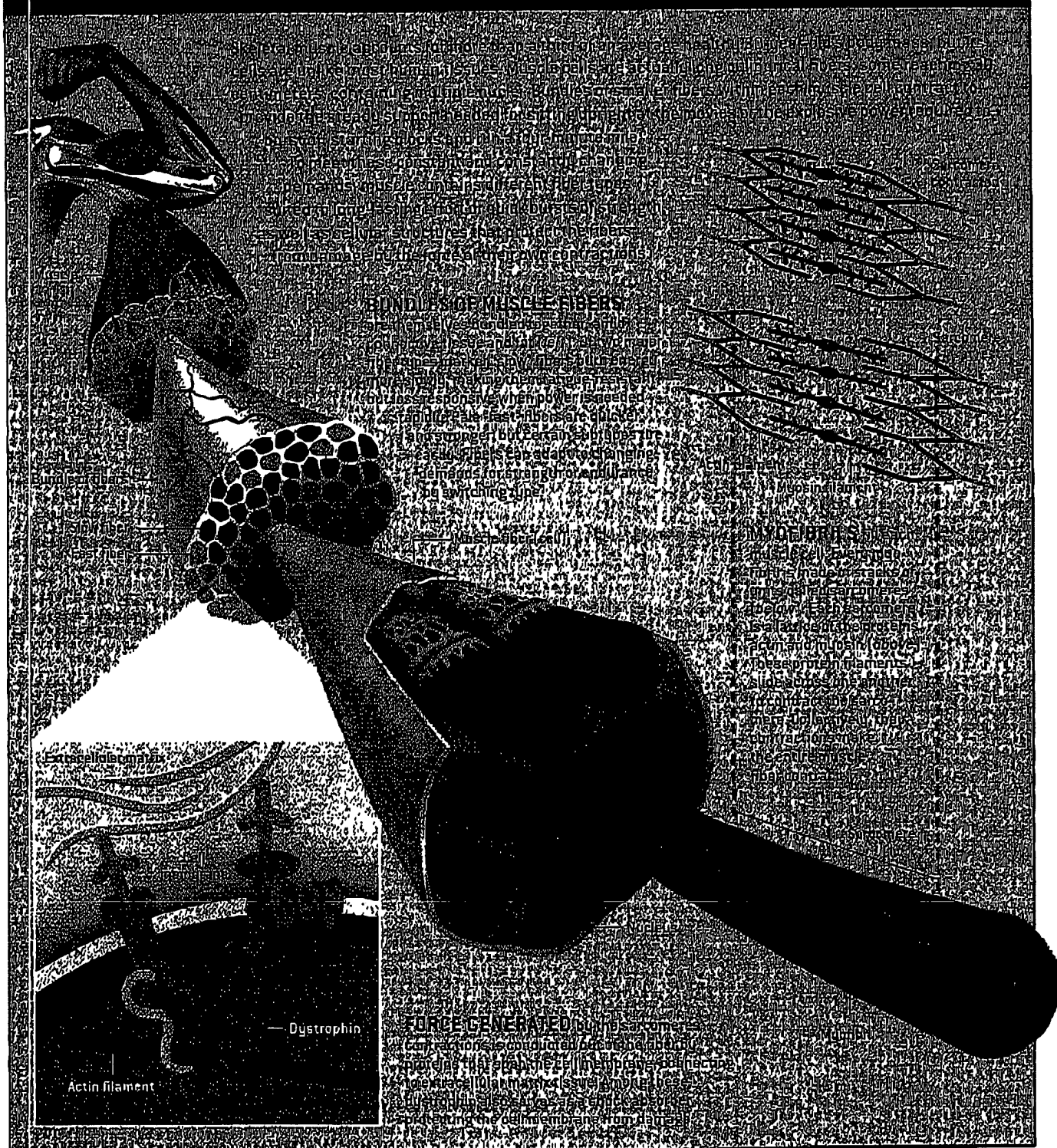
pairing themselves, although their normal regenerative mechanisms cannot keep up with the excessive rate of damage in MD. In aging muscles the rate of damage may be normal, but the repair mechanisms become less responsive. As a result, in both aging and Duchenne MD, muscle fibers die and are replaced by infiltrating fibrous tissue and fat.

In contrast, the severe skeletal muscle loss experienced by astronauts in microgravity and by patients immobilized by disability appears to be caused by a total shutdown of muscles' repair and growth mechanism at the same time apoptosis, or programmed cell death, speeds up. This phenomenon, known as disuse atrophy, is still not fully understood but makes sense from an evolutionary perspective. Skeletal muscle is metabolically expensive to maintain, so keeping a tight

Overview/Molecular Muscle Building

When a muscle fiber is damaged or stressed, it triggers a series of events that lead to the production of new muscle fibers. This process involves the activation of satellite cells, which fuse with the existing muscle fiber to form a larger, stronger fiber. The process is regulated by various growth factors, including IGF-I, which is released by the pituitary gland in response to growth hormone. IGF-I promotes the growth and differentiation of muscle cells, leading to the formation of new muscle fibers. This process is essential for muscle repair and growth, particularly in the context of aging and disease.

THE BODY'S POWERHOUSE



BUNDLES OF MUSCLE FIBERS

Each muscle fiber is a single cell, but it is not a typical cell. It is long and thin, and it is surrounded by a layer of connective tissue called the sarcolemma. The sarcolemma is made of a phospholipid bilayer, and it contains a variety of proteins, including ion channels and pumps. The sarcolemma is also responsible for the electrical coupling between adjacent muscle fibers, which allows them to contract in a coordinated manner.

MYOFIBRILS

Myofibrils are the basic contractile units of muscle. They are composed of repeating units called sarcomeres, which are made up of thick and thin filaments. The thick filaments are made of myosin, and the thin filaments are made of actin. The interaction between these two types of filaments is what generates the force of contraction.

FORCE GENERATED

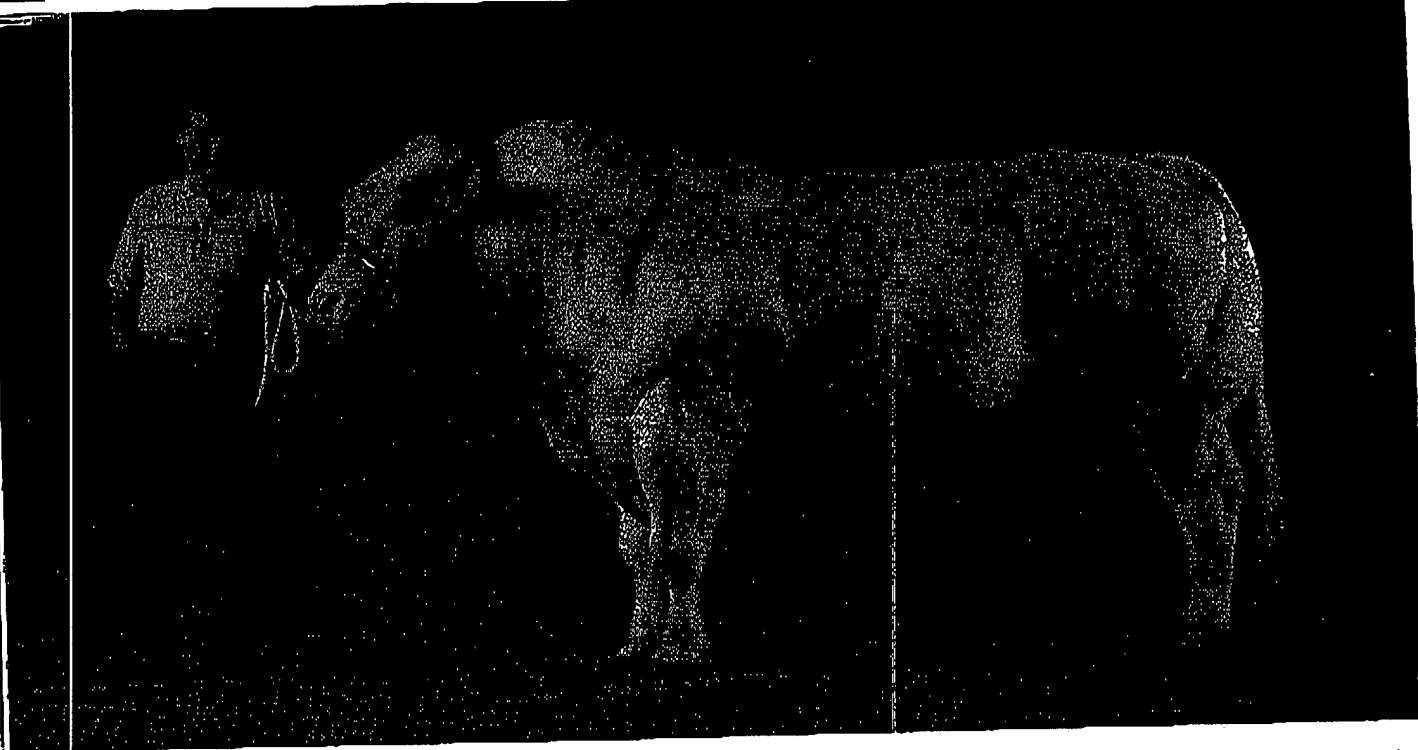
Force is generated by the interaction between the thick and thin filaments. The thick filaments have myosin heads that can bind to the thin filaments and pull them towards the center of the sarcomere. This process is called the sliding filament theory, and it is the basis of muscle contraction.

contributing their nuclei to the cell. Both pro-growth and anti-growth factors are involved in regulating this process. Satellite cells respond to insulin-like growth factor I, or IGF-I, by undergoing a greater number of cell divisions, where-

as a different growth-regulating factor, myostatin, inhibits their proliferation.

With these mechanisms in mind, about seven years ago my group at the University of Pennsylvania, in collaboration with Nadia Rosenthal and her col-

leagues at Harvard University, began to assess the possibility of using IGF-I to alter muscle function. We knew that if we injected the IGF-I protein alone, it would dissipate within hours. But once a gene enters a cell, it should keep functioning



BELGIAN BLUE BULL demonstrates the effect of blocking the antgrowth factor myostatin. A natural genetic mutation in this breed produces a truncated, ineffective form of myostatin, which allows muscle growth to go unchecked. The absence of myostatin also interferes with fat deposition, making these "double-muscled" cattle exceptionally lean.

for the life of that cell, and muscle fibers are very long-lived. A single dose of the IGF-I gene in elderly humans would probably last for the rest of their lives. So we turned our attention to finding a way to deliver the IGF-I gene directly to muscle tissue.

Donning New Genes

THEN AS NOW, a major obstacle to successful gene therapy was the difficulty of getting a chosen gene into the desired tissue. Like many other researchers, we selected a virus as our delivery vehicle, or vector, because viruses are skilled at smuggling genes into cells. They survive and propagate by tricking the cells of a host organism into bringing the virus inside, rather like a biological Trojan horse. Once within the nucleus of a host cell, the virus uses the cellular machinery to replicate its genes and produce proteins. Gene therapists capitalize on this ability by loading a synthetic gene into the virus and removing any genes the virus could use to cause disease or to replicate itself. We selected a tiny virus called adeno-associated virus (AAV) as our vector, in part because it infects human muscle readily but does not cause any known disease.

We modified it with a synthetic gene that would produce IGF-I only in skeletal

muscle and began by trying it out in normal mice. After injecting this AAV-IGF-I combination into young mice, we saw that the muscles' overall size and the rate at which they grew were 15 to 30 percent greater than normal, even though the mice were sedentary. Further, when we injected the gene into the muscles of middle-aged mice and then allowed them to reach old age, their muscles did not get any weaker.

To further evaluate this approach and its safety, Rosenthal created mice genetically engineered to overproduce IGF-I throughout their skeletal muscle. Encouragingly, they developed normally except for having skeletal muscles that ranged from 20 to 50 percent larger than those of regular mice. As these transgenic mice aged, their muscles retained a regenerative capacity typical of younger animals. Equally important, their IGF-I levels were elevated only in the muscles,

not in the bloodstream, an important distinction because high circulating levels of IGF-I can cause cardiac problems and increase cancer risk. Subsequent experiments showed that IGF-I overproduction hastens muscle repair, even in mice with a severe form of muscular dystrophy.

Raising local IGF-I production allows us to achieve a central goal of gene therapy to combat muscle-wasting diseases: breaking the close connection between muscle use and its size. Simulating the results of muscle exercise in this manner also has obvious appeal to the elite athlete. Indeed, the rate of muscle growth in young sedentary animals suggested that this treatment could also be used to genetically enhance performance of healthy muscle. Recently my laboratory worked with an exercise physiology group headed by Roger P. Farrar of the University of Texas at Austin to test this theory.

We injected AAV-IGF-I into the muscle in just one leg of each of our lab rats and then subjected the animals to an eight-week weight-training protocol. At

THE AUTHOR

H. LEE SWEENEY is professor and chairman of physiology at the University of Pennsylvania School of Medicine. He is a member of the Board of Scientific Councilors for the National Institute of Arthritis and Musculoskeletal Diseases, scientific director for Parent Project Muscular Dystrophy, and a member of the Muscular Dystrophy Association's Translational Research Advisory Council. His research ranges from basic investigation of structures that allow cells to move and generate force, particularly the myosin family of molecular motors, to translating insights about muscle cell design and behavior into gene therapy interventions for diseases, including Duchenne muscular dystrophy. He took part in a 2002 symposium on the prospect of gene doping organized by the World Anti-Doping Agency.

the end of the training, the AAV-IGF-I-injected muscles had gained nearly twice as much strength as the uninjected legs in the same animals. After training stopped, the injected muscles lost strength much more slowly than the unenhanced muscle. Even in sedentary rats, AAV-IGF-I

provided a 15 percent strength increase, similar to what we saw in the earlier mouse experiments.

We plan to continue our studies of IGF-I gene therapy in dogs because the golden retriever breed is susceptible to a particularly severe form of muscular dys-

trophy. We will also do parallel studies in healthy dogs to further test the effects and safety of inducing IGF-I overproduction. It is a potent growth and signaling factor, to which tumors also respond.

Safety concerns as well as unresolved questions about whether it is better to de-

PUMPING UP WITH GENES

By injecting a virus that carries the gene for insulin-like growth factor (IGF-I) into the muscles of a dog, researchers have found a way to increase muscle strength and size. The virus, called an adeno-associated virus (AAV), is injected into the muscle of a dog. The virus then enters the muscle cells and releases the IGF-I gene. The gene then produces IGF-I, which increases muscle strength and size. This process is called gene therapy.

The researchers found that the AAV-IGF-I-injected muscles had gained nearly twice as much strength as the uninjected legs in the same animals. After training stopped, the injected muscles lost strength much more slowly than the unenhanced muscle. Even in sedentary rats, AAV-IGF-I



GENE THERAPY The researchers found that the AAV-IGF-I-injected muscles had gained nearly twice as much strength as the uninjected legs in the same animals. After training stopped, the injected muscles lost strength much more slowly than the unenhanced muscle. Even in sedentary rats, AAV-IGF-I

provided a 15 percent strength increase, similar to what we saw in the earlier mouse experiments.

We plan to continue our studies of IGF-I gene therapy in dogs because the golden retriever breed is susceptible to a particularly severe form of muscular dys-

trophy. We will also do parallel studies in healthy dogs to further test the effects and safety of inducing IGF-I overproduction. It is a potent growth and signaling factor, to which tumors also respond.

Safety concerns as well as unresolved questions about whether it is better to de-

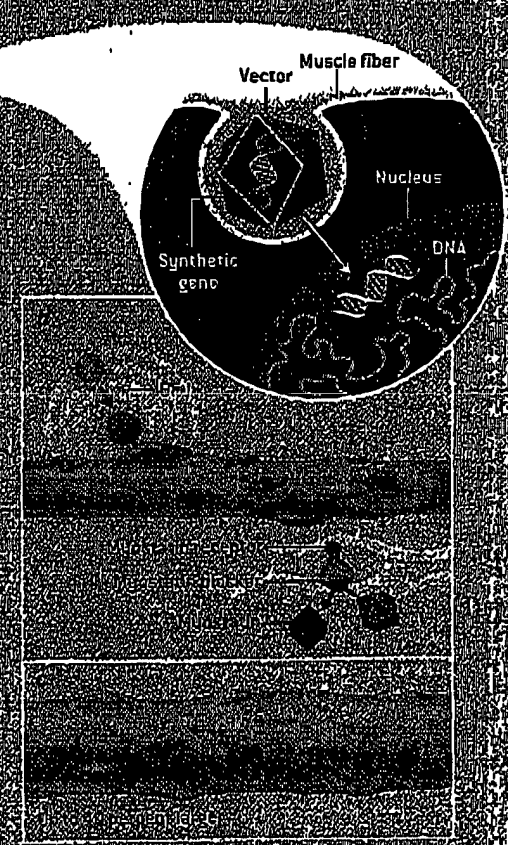
crease muscle strength and size. The virus, called an adeno-associated virus (AAV), is injected into the muscle of a dog. The virus then enters the muscle cells and releases the IGF-I gene. The gene then produces IGF-I, which increases muscle strength and size. This process is called gene therapy.

The researchers found that the AAV-IGF-I-injected muscles had gained nearly twice as much strength as the uninjected legs in the same animals. After training stopped, the injected muscles lost strength much more slowly than the unenhanced muscle. Even in sedentary rats, AAV-IGF-I

provided a 15 percent strength increase, similar to what we saw in the earlier mouse experiments.

We plan to continue our studies of IGF-I gene therapy in dogs because the golden retriever breed is susceptible to a particularly severe form of muscular dys-

trophy. We will also do parallel studies in healthy dogs to further test the effects and safety of inducing IGF-I overproduction. It is a potent growth and signaling factor, to which tumors also respond.





liver AAV in humans through the bloodstream or by direct injection into muscle mean that approved gene therapy treatments using AAV-IGF-I could be as much as a decade away. In the shorter term, human trials of gene transfer to replace the dystrophin gene are already in planning stages, and the Muscular Dystrophy Association will soon begin a clinical trial of IGF-I injections to treat myotonic dystrophy, a condition that causes prolonged muscle contraction and, hence, damage.

A still more immediate approach to driving muscle hypertrophy may come from drugs designed to block myostatin. Precisely how myostatin inhibition builds muscle is still unclear, but myostatin seems to limit muscle growth throughout embryonic development and adult life. It acts as a brake on normal muscle growth and possibly as a promoter of atrophy when functional demands on muscle decrease. Experiments on genetically engineered mice indicate that the absence of this antigrowth factor results in considerably larger muscles because of both muscle fiber hypertrophy and hyperplasia, an excessive number of muscle fibers.

Making Muscle and More

PHARMACEUTICAL AND biotechnology companies are working on a variety of myostatin inhibitors. Initially, the possibility of producing meatier food animals piqued commercial interest. Nature has already provided examples of the effects of myostatin blockade in the Belgian Blue and Piedmontese cattle breeds, both of which have an inherited genetic mutation that produces a truncated, ineffective version of myostatin. These cattle are often called double-muscling, and their exaggerated musculature is all the more impressive because an absence of myostatin also interferes with fat deposition, giving the animals a lean, sculpted appearance.

The first myostatin-blocking drugs to have been developed are antibodies against myostatin, one of which may soon undergo clinical testing in muscular dystrophy patients. A different approach mimics the cattle mutation by creating a smaller version of myostatin, which lacks

the normal molecule's signaling ability while retaining the structures that dock near satellite cells. This smaller protein, or peptide, essentially caps those docking locations, preventing myostatin from attaching to them. Injecting the peptide into mice produces skeletal muscle hypertrophy, and my colleagues and I will be attempting to create the same effect in our dog models by transferring a synthetic gene for the peptide.

Myostatin-blocking therapies also have obvious appeal to healthy people seeking rapid muscle growth. Although systemic drugs cannot target specific muscles, as gene transfer can, drugs have the benefit of easy delivery, and they can immediately be discontinued if a problem arises. On the other hand, such drugs would be relatively easy for sport regulatory agencies to detect with a blood test.

But what if athletes were to use a gene therapy approach similar to our AAV-IGF-I strategy? The product of the gene would be found just in the muscle, not in the blood or urine, and would be identical to its natural counterpart. Only a muscle biopsy could test for the presence of a particular synthetic gene or of a vector. But in the case of AAV, many people may be naturally infected with this harmless virus, so the test would not be conclusive for doping. Moreover, because most athletes would be unwilling to undergo an invasive biopsy before a competition, this type of genetic enhancement would remain virtually invisible.

And what of the safety of rapidly increasing muscle mass by 20 to 40 percent? Could an athlete sporting genetically inflated musculature exert enough force to snap his or her own bones or tendons? Probably not. We worry more about building muscle in elderly patients with bones weakened by osteoporosis. In a healthy young person, muscle growth occurring over weeks or months would give supporting skeletal elements time to grow to meet their new demands.

This safety question, however, is just one of the many that need further study in animals before these treatments can even be considered for mere enhancement of healthy humans. Nevertheless, with gene therapy poised to finally be-

come a viable medical treatment, gene doping cannot be far behind, and overall muscle enlargement is but one way that it could be used [see illustration on page 67]. In sports such as sprinting, twacking genes to convert muscle fibers to the fast type might also be desirable. For a marathoner, boosting endurance might be paramount.

Muscle is most likely to be the first tissue subject to genetic enhancement, but others could eventually follow. For example, endurance is also affected by the amount of oxygen reaching muscles. Erythropoietin is a naturally occurring

regularly diluted to keep their hearts from failing.

The technology necessary to abuse gene transfer is certainly not yet within reach of the average athlete. Still, officials in the athletic community fear that just as technically skilled individuals have turned to the manufacture and sale of so-called designer steroids, someday soon a market in genetic enhancement may emerge. Policing such abuse will be much harder than monitoring drug use, because detection will be difficult.

It is also likely, however, that in the decades to come, some of these gene ther-

With gene therapy poised to become a viable medical treatment, gene doping cannot be far behind.

protein that spurs development of oxygen-carrying red blood cells. Its synthetic form, a drug called Epoetin, or simply EPO, was developed to treat anemia but has been widely abused by athletes—most publicly by cyclists in the 1998 Tour de France. An entire team was excluded from that race when their EPO use was uncovered, yet EPO abuse in sports continues.

Gene transfer to raise erythropoietin production has already been tried in animals, with results that illustrate the potential dangers of prematurely attempting such enhancements in humans. In 1997 and 1998 scientists tried transferring synthetic erythropoietin genes into monkeys and baboons. In both experiments, the animals' red blood cell counts nearly doubled within 10 weeks, producing blood so thick that it had to be

apics will be proved safe and will become available to the general population. If the time does arrive when genetic enhancement is widely used to improve quality of life, society's ethical stance on manipulating our genes will probably be much different than it is today. Sports authorities already acknowledge that muscle-regenerating therapies may be useful in helping athletes to recover from injuries.

So will we one day be engineering superathletes or simply bettering the health of the entire population with gene transfer? Even in its infancy, this technology clearly has tremendous potential to change both sports and our society. The ethical issues surrounding genetic enhancement are many and complex. But for once, we have time to discuss and debate them before the ability to use this power is upon us.

MORE TO EXPLORE

Viral Mediated Expression of Insulin-like Growth Factor I Blocks the Aging-Related Loss of Skeletal Muscle Function. Elisabeth R. Barton-Davis et al. in *Proceedings of the National Academy of Sciences USA*, Vol. 95, No. 26, pages 15,603–15,607; December 22, 1998.

Muscle, Genes and Athletic Performance. Jasper L. Anderson, Peter Schjerling and Bengt Saitin in *Scientific American*, Vol. 283, No. 3, pages 48–55; September 2000.

Toward Molecular Talent Scouting. Gary Taubes in *Scientific American Presents: Building the Elite Athlete*, Vol. 11, No. 3, pages 26–31; Fall 2000.

Viral Expression of Insulin-like Growth Factor-I Enhances Muscle Hypertrophy in Resistance-Trained Rats. Sukho Lee et al. in *Journal of Applied Physiology*, Vol. 96, No. 3, pages 1097–1104; March 2004.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☒ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☒ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.